



## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(51) International Patent Classification<sup>6</sup> :</b> <b>A61K 7/06</b>	<b>A1</b>	<b>(11) International Publication Number:</b> <b>WO 98/02134</b> <b>(43) International Publication Date:</b> 22 January 1998 (22.01.98)
<b>(21) International Application Number:</b> PCT/US97/11033 <b>(22) International Filing Date:</b> 25 June 1997 (25.06.97) <b>(30) Priority Data:</b> 60/021,629 12 July 1996 (12.07.96) US <b>(71) Applicant (for all designated States except US):</b> JOHNSON & JOHNSON CONSUMER PRODUCTS, INC. [US/US]; Grandview Road, Skillman, NJ 08558 (US). <b>(72) Inventors; and</b> <b>(75) Inventors/Applicants (for US only):</b> SEIBERG, Miri [IL/US]; 168 Herrontown Road, Princeton, NJ 08540 (US). SHAPIRO, Stanley, S. [US/US]; 10 Plymouth Drive, Livingston, NJ 07039 (US). CAUWENBERGH, Gerard, F., M., J. [BE/US]; 10 Beechtree Lane, Plainsboro, NJ 08536 (US). WISNIEWSKI, Stephen, J. [US/US]; 6280 Pt. Pleasant Pike, Doylestown, PA 18901 (US). <b>(74) Agents:</b> CIAMPORCERO, Audley, A. et al.; Johnson and Johnson, One Johnson & Johnson Plaza, New Brunswick, NJ 08933-7003 (US).		<b>(81) Designated States:</b> AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, ARIPO patent (GH, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).  <b>Published</b> <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>
<b>(54) Title:</b> METHODS FOR ALTERING HAIR GROWTH AND HAIR PIGMENTATION BY APOPTOSIS IN THE FOLLICULAR PAPILLAE AND COMPOSITIONS THEREFOR		
<b>(57) Abstract</b> <p>The present invention utilizes serine proteases and their ability to induce programmed cell death and apoptosis in the follicular papillae to affect changes in mammalian hair growth and hair pigmentation. Also described are compositions which have an agent with a portion of similar structure to a portion of the trypsin molecule, allowing said agent to induce programmed cell death and apoptosis in the same manner as trypsin.</p> <div style="display: flex; justify-content: space-around; align-items: flex-end;"> <div style="text-align: center;"> <p>Fig. 10a</p> </div> <div style="text-align: center;"> <p>Fig. 10b</p> </div> </div> <div style="text-align: center; margin-top: 20px;"> <p>Fig. 10c</p> </div>		

**FOR THE PURPOSES OF INFORMATION ONLY**

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece			TR	Turkey
BG	Bulgaria	HU	Hungary	ML	Mali	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MN	Mongolia	UA	Ukraine
BR	Brazil	IL	Israel	MR	Mauritania	UG	Uganda
BY	Belarus	IS	Iceland	MW	Malawi	US	United States of America
CA	Canada	IT	Italy	MX	Mexico	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NE	Niger	VN	Viet Nam
CG	Congo	KE	Kenya	NL	Netherlands	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NO	Norway	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	NZ	New Zealand		
CM	Cameroon	KR	Republic of Korea	PL	Poland		
CN	China	KZ	Kazakhstan	PT	Portugal		
CU	Cuba	LC	Saint Lucia	RO	Romania		
CZ	Czech Republic	LI	Liechtenstein	RU	Russian Federation		
DE	Germany	LK	Sri Lanka	SD	Sudan		
DK	Denmark	LR	Liberia	SE	Sweden		
EE	Estonia			SG	Singapore		

5

10

**Methods for Altering Hair Growth and Hair Pigmentation by Apoptosis  
in the Follicular Papillae and Compositions Therefor**

Cross-Reference to Related Application

15

This Application claims the benefit of United States Provisional Application Number 60/021,829 filed on 12 July 1996, which is incorporated by reference in its entirety.

Field of the Invention

20

This invention is related to methods for bringing about programmed cell death and apoptosis in the follicular papillae and compositions effective for the same. More specifically, the present invention is directed to methods for changing the rate of hair growth and hair pigmentation by topical application of either a protease or a molecule that affects a receptor-mediated signal transduction pathway.

25

Background of the Invention

One main function of mammalian hair is to provide environmental protection. However, that function has largely been lost in humans, in whom hair is kept or removed essentially for cosmetic purposes.

30

Many procedures are used to remove unwanted hair including shaving, electrolysis, plucking and injection of therapeutic antiandrogens. These conventional methods are not without their shortcomings. Shaving, for instance, may result in nicks and cuts in the skin's surface, may leave a perception of an increase in the rate of hair regrowth, and may also leave an

5       undesirable stubble. While electrolysis may keep an area free of unwanted hair for a prolonged period of time, the process is often expensive and painful and may further result in scarring. Not only may plucking cause pain and discomfort, but it often results in a poor removal of short hair. Several unwanted side effects, such as effects on muscularity, often accompany the use of antiandrogens. Furthermore, with the exception of electrolysis, all of these methods must be repeated on what is often a daily basis, creating an inconvenience for the user. For these reasons, chemical methods for delaying hair growth are needed.

10       As disclosed in U.S. Patent No. 5,455,234 by Ahluwalia, et al, mammalian hair growth may be altered by applying a cysteine pathway enzyme inhibitor to the skin, which will slow or stop the metabolic pathways for rate and character of hair growth with which cysteine is associated. However, this inhibition of cysteine also causes a shortage of this amino acid in the treated area. Similarly, in U.S. Patent No. 5,095,007 by Ahluwalia, U.S. Patent Nos. 5,096,911 and 5,468,476 by Ahluwalia *et al.* and U.S. Patent Nos. 5,132,293 and 5,143,925 by Shander *et al.*, enzyme inhibitors are utilized to alter hair growth. In each of these cases a biochemical pathway was altered to inhibit the production of a final product.

20       It would be desirable to provide a method for chemically affecting hair growth and hair pigmentation which does not deprive the user of needed amino acids or cause unwanted side effects to the user.

#### Summary of the Invention

25       In accordance with the present invention, we have found a method for affecting changes in mammalian hair growth and hair pigmentation comprising, consisting essentially of, or consisting of:

5           topically applying to skin of a mammal an effective amount of a  
topically active composition comprising a protease within a time sufficient  
relative to telogen hair growth induction.

          In another embodiment of the present invention, we have found a  
method for inducing programmed cell death and apoptosis within a follicular  
10       papilla comprising, consisting essentially of, or consisting of:

          topically applying to skin of a mammal a topically active agent having  
a first portion which is similar in shape to a second portion of a three-  
dimensional structure of trypsin, whereby the first portion and the second  
portion are both capable of receptor-mediated signal transduction.

15       In another embodiment of the present invention, we have found a  
composition comprising, consisting essentially of, or consisting of:

          a) a topically active agent having a first portion which is similar in  
shape to a second portion of a three-dimensional structure of trypsin,  
whereby the first portion and the second portion are both capable of  
20       receptor-mediated signal transduction; and

          b) a pharmaceutically or cosmetically acceptable vehicle.

          In yet another embodiment of the present invention, we have found a  
composition comprising, consisting essentially of, or consisting of:

          a) a topically active agent comprising a protease capable of inducing  
25       apoptosis in a follicular papilla; and

          b) a pharmaceutically or cosmetically acceptable vehicle.

          The compositions and methods of this invention provide a unique,  
convenient means for delaying hair growth and hair pigmentation by  
inducing apoptosis at the follicular papillae.

30       Brief Description of the Drawings

5           The file of this patent contains at least one drawing executed in color. Copies of this patent with color drawing(s) will be provided by the Patent and Trademark Office upon request and payment of the necessary fee.

          The invention will be more fully understood and further advantages will become apparent when reference is made to the following detailed  
10 description of the invention and the accompanying drawings in which:

          FIGS. 1 (a) and 1(c) are representations which illustrate a cross-sectional view of the skin of an untreated C57Bl/6 mouse labeled with fluorescent trypsin. FIG. 1(b) is a representation which illustrates a cross-sectional view of the skin of a C57Bl/6 mouse 12 days post-topical treatment  
15 with trypsin mouse labeled with fluorescent trypsin.

          FIG. 2 are color representations of the dorsal view of the skin of C57Bl/6 mice, some of which were treated with vehicle or trypsin (1%) immediately after induction for hair growth, taken: (a) 8 days after hair growth induction; (b) 11 days after hair growth induction; and (c) 14 days  
20 after hair growth induction. In FIG. 2(a), the left mouse was treated, while the right mouse was untreated. In FIGS. 2(b) and 2(c), the left mouse was untreated, the center mouse was treated with the vehicle and right mouse was treated with trypsin.

          FIG. 3A is a representation which illustrates the histology of hair  
25 follicles of untreated mouse skins at: (a) 4 days; (b) 5 days; (c) 8 days; and (d) 12 days post hair induction. FIG. 3B is a representation which illustrates the histology of hair follicles of trypsin-treated mouse skins at: (a) 4 days; (b) 5 days; (c) 6 days; (d,e) 8; and (f) 12 days post hair induction.

          FIG. 4(A) is a representation which illustrates the cross-sectional  
30 view of TUNEL-stained skin tissue of an untreated mouse at : (a) 1 day; (b) 2 days; and (c) 3 days post hair induction. FIG. 4(B) is a representation which illustrates the cross-sectional view of TUNEL-stained skin tissue of a trypsin-treated mouse at: (a) 1 day; (b) 2 days; (c) 3 days; (d) 4 days; (e) 5

5 days post hair induction ((a - e) were single trypsin applications); and (f) 8 days post hair induction (daily trypsin applications).

FIG. 5 is a representation which illustrates the profile of gene expression of a trypsin-treated mouse skin and an untreated mouse skin during a delayed hair cycle as detected by Reverse Transcription-Polymer Chain Reaction ("RT-PCR"). FIG. 5(A) illustrates the mRNA levels throughout days 1 to 11 of the delayed hair cycle. FIG. 5(B) illustrates the mRNA levels at day 8 of the delayed hair growth cycle. The RT-PCR products of 25ng (A) and 250ng (B) of total RNA are shown.

#### 15 Detailed Description of the Invention

As used herein, "mammal" shall mean any member "of the higher vertebrae animals comprising the class Mammalia," as defined in Webster's Medical Desk Dictionary 407 (1986), and includes but is not limited to humans. As used herein "(%, w/v)" shall mean grams of a given component per 100 ml of the total composition. As used herein, "receptor" shall include both intracellular and extracellular receptors and shall mean those molecules capable of receiving and transducing a signal.

20 Topically active agents suitable for use in the composition of the present invention include proteases, compounds having a structure substantially similar to a portion of the three-dimensional structure of trypsin, or mixtures thereof.

25 Preferred proteases are the serine proteases which include, but are not limited to, trypsin, carboxypeptidase-Y, protease IV, subtilysin, or mixtures thereof. The protease of choice is trypsin. While not willing to be bound by this theory, we believe that the proteases capable of delaying hair growth and hair pigmentation do so by altering the hair growth cycle via the induction of programmed cell death and apoptosis within the follicular papillae, and that this alteration is likely induced by a receptor-mediated

30

5 signal transduction. Preferably, the protease is present in an amount, based upon the total volume of the composition of the present invention, of from about 0% (w/v) to about 5% (w/v), and more preferably from about 0.01% (w/v) to about 1% (w/v).

10 As evidenced by Example 10 herein, we have unexpectedly found that the proteolytic activity of a protease is not the only factor which contributes to the induction of programmed cell death. While not wishing to be bound by any theory, we further believe that a receptor-mediated signal transduction event may also contribute to the induction of programmed cell death of the follicular papillae, and that this type of agent might be related to  
15 a structural component of the topically active agent.

Therefore, a second suitable topically active agent includes compounds which possess a portion that is substantially similar in shape to the portion(s) of a three-dimensional structure of a trypsin molecule that affect this receptor-mediated signal transduction. As used herein,  
20 "substantially similar in shape" means that the compounds include a sufficient amount of a structure behaving like a ligand which either may occupy a receptor, may displace a ligand from the receptor, or may activate or inactivate a receptor by a proteolytic cleavage, and thereby contribute to the induction of programmed cell death. Examples of such compounds  
25 possessing portions which are substantially similar in shape to the structure of a portion of three-dimensional trypsin include, but are not limited to proteolytically inactive trypsin, which is a form of trypsin that is unable to digest proteins such as collagen. Preferably these compounds are present in the composition of the present invention in an amount, based upon the  
30 total volume of the composition, of from about 0% (w/v) to about 5% (w/v) and most preferably from about 0.01% (w/v) to about 1% (w/v).

If the delivery parameters of the topically active pharmaceutical or cosmetic agent so require, the topically active composition of the present



5 invention may preferably be further comprised of a pharmaceutically or  
cosmetically acceptable vehicle capable of functioning as a delivery system  
to enable the penetration of the topically active agent into the hair follicle.  
While any commercially available vehicle for delivering the protease to the  
appropriate skin appendage, which in this case is the hair follicle, is suitable  
10 for use as the pharmaceutically or cosmetically acceptable vehicle,  
liposomes are preferred. The liposomes are more preferably non-ionic and  
comprised of a) glycerol dilaurate or glycerol distearate; b) compounds  
having the steroid backbone found in cholesterol; and c) fatty acid ethers  
having from about 12 to about 18 carbon atoms, wherein the constituent  
15 compounds of the liposomes are in a ratio of about 53: 10: 22 to about 63:  
20: 32 , and preferably from about 55:12:24 to about 61:18:30, respectively.  
Liposomes comprised of glycerol dilaurate / cholesterol/ polyoxyethylene -  
10-stearyl ether (GDL) are most preferred. Preferably the liposomes are  
present in an amount, based upon the total volume of the composition, of  
20 from about 10 mg/mL to about 100 mg/mL, and more preferably from about  
25 mg/mL to about 50 mg/mL. A ratio of about 58:15:27 is most preferred.  
Suitable liposomes may preferably be prepared in accordance with the  
protocol set forth in Example 2, though other methods commonly used in the  
art are also acceptable.

25 The above described composition may be prepared by combining the  
desired components in a suitable container and mixing them under ambient  
conditions in any conventional high shear mixing means well known in the art  
for non-ionic liposomes preparations, such as those disclosed in Niemiec *et al.*,  
"Influence of Nonionic Liposomal Composition On Topical Delivery of  
30 Peptide Drugs Into Pilosebaceous Units: An In Vivo Study Using the  
Hamster Ear Model," 12 Pharm. Res. 1184-88 (1995) ("Niemiec"), which is  
incorporated by reference herein in its entirety.

5           In a preferred embodiment, the pH of the topically active pharmaceutical or cosmetic composition may be adjusted with a known pH adjuster including but not limited to N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid available from, for example, Life Technologies under the tradename "Hepes" , or (hydroxymethyl) aminomethane, available from, for  
10           example, Life Technologies under the tradename, "Tris" , citric acid, and mixtures thereof to yield a pH of from about 2 to about 8 in the final composition.

          In alternative embodiments, the topically active pharmaceutical or cosmetic composition may be optionally combined with other ingredients  
15           such as moisturizers, foaming agents, cosmetic adjuvants, anti-oxidants, surfactants, foaming agents, conditioners, humectants, fragrances, viscosifiers, buffering agents, preservatives, and the like in an amount which will not destroy the liposomal structure in order to produce cosmetic or pharmaceutical products such as, non-exclusively, shaving creams, shaving  
20           gels, shaving powders, chemical depilatory creams and the like.

          In another embodiment of the present invention, we have found a process for affecting changes in mammalian hair growth and hair pigmentation comprised of applying the topically active pharmaceutical or cosmetic composition to the surface of an animal's skin at about the time of  
25           hair growth induction.

          Hair growth induction from telogen follicles can be performed by any method well known in the art, including, but not limited to, shaving, wax depilation, chemical depilation and combinations thereof.

          Although the time at which the topically active pharmaceutical or  
30           cosmetic composition is applied to the skin as well as the amount of time for which the composition remains on the skin may vary, one skilled in the art would readily realize without undue experimentation that the topically active composition is preferably applied to the skin surface either immediately

5 before, immediately after or simultaneously with hair growth induction from a telogen state. As used herein, "immediately" shall be defined as within a period of about one hour. More preferably, the topically active pharmaceutical or cosmetic composition is applied either simultaneously with or immediately following hair growth induction and is left on the skin for  
10 a period sufficient to delay hair growth, which is preferably at least about five minutes and more preferably is at least about fifteen minutes after the time of application.

The topically active pharmaceutical or cosmetic composition should be applied in an amount effective to affect changes in mammalian hair  
15 growth and hair pigmentation. As used herein "amount effective" shall mean an amount sufficient to cover the region of skin surface where a delay in hair growth and hair pigmentation is desired. Preferably, the composition is applied to the skin surface such that, based upon a square cm of skin surface, from about 2  $\mu\text{l}/\text{cm}^2$  to about 8  $\mu\text{l}/\text{cm}^2$  of topically active agent is  
20 present when a delay in hair growth and hair pigmentation is desired.

We have unexpectedly found that when topically active agents, such as serine proteases, are topically applied to an animal's skin at about the time of hair growth induction, a significant delay in hair growth and hair  
25 pigmentation of at least about 9 days was achieved. We further believe that since the hair growth cycle for humans is often slower than that for mice, it is further likely that the hair growth delay in humans would be considerably longer than 9 days.

The invention illustratively disclosed herein suitably may be practiced in the absence of any component, ingredient, or step which is not specifically  
30 disclosed herein. Several examples are set forth below to further illustrate the nature of the invention and the manner of carrying it out. However, the invention should not be considered as being limited to the details thereof.

5 Examples

**EXAMPLE 1: Depilation of Test Subjects in the Mouse System**

The hair growth of 12 C57Bl/6 female mice obtained from Charles River (Kingston, NY), which were 6-10 weeks of age and were in the resting (telogen) phase of their respective hair cycle, was induced by wax depilation (plucking) of each respective animal's back fur according to the procedure set forth in Stenn, et al., "Glucocorticoid Effect on Hair Growth Initiation: A Reconsideration," *6 Skin Pharmacol.*, 125 -134 (1993). As well-known in mice, the growth phase (anagen) starts synchronously in all hair follicles at the time of depilation.

15 As illustrated in Table 1, the following observations were noticed at the induction site:

**Table 1: Observations at Induction Site**

<u>Days Post-Induction</u>	<u>MORPHOLOGICAL AND Histological Observations at the induction site</u>
1 - 2 (early anagen)	new follicle starts to grow
3 to 4	hair follicles were fully developed, but the hair shafts were not yet visible
8 (late anagen)	each mouse had very dark skin; their hair shafts are histologically visible
11 - 12	the hair shafts started to penetrate through the epidermis.
14	each mouse was covered with short hairs
19	the regression of the follicle (catagen) was observed histologically
21 to 25	the hair follicle is back to resting phase.

20 As shown in Table 1, the hair growth was visible several days after depilation as the pink skin of the animal began to darken. This is likely due

5 to hair pigmentation in the shaft since the C57Bl/6 mouse contained melanocytes only in the hair follicles and not in the epidermis.

The hair growth from telogen of 12 similar C57Bl/6 mice was also induced by chemical depilation with a hair remover available from Carter-Wallace, Incorporated under the tradename "Nair" in an amount sufficient to  
10 wet the back of each mouse. In chemical depilation, the lower portion of the follicles remained intact and lower portions of hair shafts of the previous cycle remained intact in the dermis until pushed out by the newly-grown hairs. The growth pattern of the new hair cycle was identical to the one described in Table 1.

15 Since the murine hair cycle varies not only between strains, but also amongst individual animals, a 2 cm by 1 cm skin sample was isolated from each mouse with scissors, fixed with a 10% buffered formalin solution having a pH of about 6.9 - 7.1 at 25 °C available from Stephens Scientific, and then formed into a paraffin block according to well-known procedures.  
20 The block was then microtomed, stained with H&E stain, and examined histologically in order to verify the phase of the hair cycle using procedures well-known in the art. The histological representations obtained from this Example are illustrated in FIG. 3A.

This Example shows that the hair growth cycle for C57Bl/6 mice  
25 averaged about 25 days and reports similar timing of hair follicle and shaft development regardless of the method used for depilation.

#### **EXAMPLE 2: Preparation of Topically Active Compositions**

A sufficient amount of lyophilized trypsin available from Sigma-  
30 Aldrich Corporation was mixed into a buffered aqueous solution of 0.05 M N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid available from Life Technologies under the tradename "Hepes" such that the pH of the resulting solution was about 7.4 and the concentration of trypsin in the solution was

5        about 2% (w/v). 1 volume of the resulting trypsin solution was then mixed  
with 1 volume of (5 %) glycerol dilaurate/cholesterol/polyoxyethylene-10-  
stearyl ether liposomes in water vehicle, which was prepared by the  
methods described in Niemiec, in order to yield a 1% (w/v) concentration of  
trypsin in the resulting topically active composition.

10        **EXAMPLE 3: Delivery of Trypsin into Hair Follicles**

Twelve days after plucking the hairs from about a 6 cm by 2 cm  
section of the skin surface of 4 C57Bl/6 female, 7 to 8 week old mice  
available from Charles River (Kingston, NY), about 100  $\mu$ L/mouse of the  
15        topically active composition of Example 2 was applied thereto. The trypsin  
used in this Example was fluorescently-labeled with a FluoReporter protein  
labelling kit available from Molecular Probes, Inc. in accordance with its  
accompanying protocol (1997) and using a fluorescein isothiocyanate  
(FITC) fluorescent label, also available from Molecular Probes, Inc.

20        At one and four hours after the application of the fluorescent trypsin  
treatment, a 2 cm x 1 cm sample of the skin surface of each mouse was  
isolated and formed into a paraffin block which was then microtomed and  
examined histologically in accordance with the procedure set forth in  
Example 1.

25        As shown in FIG. 1, almost all of the fluorescent labeling was found  
within the hair follicle. The mice were examined at the 1 hour (FIG. 1(c))  
interval and the 4 hour (FIG. 1(a)) interval displayed identical histological  
staining patterns, with no additional skin penetration at the later time point.  
This observation suggests against a possible non-specific extracellular  
30        matrix digestion by the protease, which would have likely shown a deeper  
penetration of the fluorescent stain into the stratum corneum at the later  
time point.

5           This Example was repeated on 4 similar C57Bl/6 female, 7 to 8 week old mice, but with the exception that the mice were also treated daily for 12 days post-induction with the 1% (w/v) trypsin treatment of Example 2. Four hours after the application of the fluorescent-trypsin treatment on the twelfth day post-induction, the skins of the mice were analyzed using similar  
10           histological methods. As illustrated in FIG. 1b, no major change was observed in the delivery route of the trypsin into the hair follicles of the treated skins. However, the minimal staining at the outer portion of the stratum corneum of the trypsin-treated skins indicated some loss of barrier integrity.

15           This Example shows that the application of a topically active composition containing trypsin to the skin surface of anagen animals resulted in the delivery of the trypsin primarily to the hair follicle, both after short term and long term use.

20           **EXAMPLE 4: Trypsin Delays Hair Growth and Hair Pigmentation**

          A single topical application of the topically active composition prepared in Example 2 was applied to 12 C57Bl/6 female, 7 to 8 week old mice immediately after hair growth induction in accordance with the method set forth in Example 3.

25           As shown in FIG. 2, a single application of trypsin applied post-hair growth induction delayed both hair growth and hair pigmentation. The darker skin color indicated a more progressive stage of the hair cycle, which is before the hair shafts were visible. Untreated and liposome (vehicle)-treated controls exhibited dark skin at 7-8 days post-hair growth induction (FIG. 2a; left is treated and right is untreated), while their hair shafts were  
30           visible at 11-13 days (FIG. 2b; left is untreated; middle is vehicle; right is treated). By contrast, the trypsin-treated animals remained pink until day 8 (FIG. 2a; left mouse); were slightly darker pink at day 11 (FIG. 2b; right

5 mouse); and exhibited gray skin by day 14 (FIG. 2c; right mouse). Although the hair shafts of the trypsin-treated animals were first visible at days 15 to 17, these hair shafts were of reduced quality and were not as thick as in the control; however, within about 4 to 7 additional days, these hair shafts were almost indistinguishable from those in the controls in all characteristics  
10 except for length.

This Example shows that trypsin was effective for delaying both hair growth and hair pigmentation.

**EXAMPLE 5: Histology of Trypsin Treated Hair Follicles**

15 A single topically active composition as prepared in Example 2 was applied to the skin surface of 3 C57BL/6 female, 7 to 8 week old mice after hair induction in accordance with the method set forth in Example 3.

1 cm x 2 cm samples of the skins of untreated, vehicle-treated (not shown), and trypsin-treated mice were obtained, H&E stained, then  
20 examined histologically via the procedure set forth in Example 1. As illustrated in FIG. 3, histological analysis of the stained samples evidenced that the development of the hair follicles in trypsin-treated mice was significantly delayed. More specifically, the characteristic layered structure, the expanded follicular papillae and the new hair pigmentation were first  
25 observed several days later than the date of first observation of such characteristics in the untreated and vehicle-treated controls. In addition, the follicles in the skin of trypsin-treated mice displayed a dilated infundibulum, as best illustrated in FIG. 3B(a-e), which was not present in the untreated and the vehicle-treated mice as shown in FIG. 3A(a-b). This  
30 observation supports the fact that treatment with the trypsin-containing composition also resulted in hair growth having a reduced quality, i.e. thinner and more sparse. At 7-8 days post- treatment with trypsin, the lower part of some treated follicles started to form an epithelial layering



5 structure as illustrated in FIG. 3B(d - e), but the upper part still displayed an empty structure as best shown in FIG. 3B(d). By 11-12 days post-treatment with trypsin, most of the treated follicles matured, but still displayed reduced hair pigmentation and shorter shaft length that resembled the characteristics of the untreated follicle at 4 to 5 days post-induction (compare FIG. 3A(d) to  
10 FIG. 3B(f)).

This Example further showed via histological analysis that the topical application of trypsin to the skin of hair growth induced mice significantly delayed hair growth and hair pigmentation.

15 **EXAMPLE 6: Trypsin Induces Apoptosis at the Follicular Papillae**

A single topically active composition as prepared in Example 2 was applied to the skin surface of 3 C57BL/6 female, 7 to 8 week old mice after hair induction in accordance with the method set forth in Example 3.

1 cm x 2 cm samples of the skins of untreated and trypsin-treated  
20 mice were obtained via the procedure set forth in Example 1 then analyzed via a TdT-mediated dUTP-biotin nick end labeling ("TUNEL") procedure as disclosed in Gavrieli et al., "Identification of Programmed Cell Death in situ Via Specific Labelling of Nuclear DNA Fragmentation", 119 J. Cell Biology 493-501 (1992)("Gavrieli"). During this procedure, the prepared paraffin  
25 sections of the skins were stained using an "ApopTag™ Plus InSitu Apoptosis Detection Kit" available from Oncor, Inc. as specified in the "ApopTag™ Plus InSitu Apoptosis Detection Kit" protocol by Oncor, Inc. (Feb. 1995), which is based upon the labeling of fragmented DNA ends as described in Gavrieli. FIG. 4 shows a histological analysis wherein the  
30 stain has a peroxidase end point (brown) and a methyl green counter-stain.

Similarly, paraffin sections suitable for TUNEL staining were prepared from the untreated skins of mice. These sections were stained

5 and analyzed histologically; the resulting representations of which are provided in FIG. 4A(a-c).

As illustrated in FIG. 4, the TUNEL-stained samples defined apoptotic cells by both morphology (condensed or fragmented nuclei and cytoplasm or apoptotic bodies) and by the color of its stain (fragmented DNA within the condensed nuclei were stained brown). More specifically, FIG. 4B(a - e) illustrated that apoptotic bodies were detected within the treated follicular papilla throughout the first week after hair growth induction. However, no other portions of the follicle, epidermis or dermis were affected by the trypsin treatment. By contrast, a minimal level of apoptosis was occasionally detected in untreated early anagen follicles as illustrated in the TUNEL-stained untreated sample in FIG. 4A(a-c); however, the apoptosis was always at the isthmus of the follicle and thus well above the follicular papilla. Most of the untreated follicles did not display any cell death.

This Example showed that the application of trypsin to depilated skin resulted in an increase in apoptotic figures within the follicular papillae of the trypsin treated follicles, relative to that of either untreated or vehicle treated controls.

25 ***EXAMPLE 7: Trypsin Induces Changes in  
Gene Expression During the Hair Cycle***

A single topically active composition as prepared in Example 2 was applied to the skin surface of 6 C57BL/6 female, 7 to 8 week old mice after hair induction in accordance with the method set forth in Example 3.

30 At the time intervals indicated in FIG. 5, the skins of untreated mice and trypsin-treated mice were obtained as described in Example 1, then their total RNAs were extracted using "RNA Stat-60" reagent available from Tel-Test "B," Incorporated as described in Chomczynski, "Single Step Method of RNA Isolation By Acid Guanidinium Thiocyanate-phenol-

chloroform extraction," 162 Anal. Biochem. 156-59 (1987). A sufficient amount of RNase-free DNase available from Promega, Corporation under the tradename "RQ1 RNase-free DNase" was then added to the extracted RNA from each mouse such that each respective product contained 200ng of DNase-RNA using the procedure set forth in "RNase-free DNase", protocol published by Promega, Corporation (May, 1995). The resulting 200ng of DNase-RNA was reverse transcribed ("RT") via the procedure set forth in "Superscript II Reverse Transcriptase" a protocol published by Gibco-BRL (now Life Technologies, Incorporated) (April 1992), using random hexamers such as the random primers which are commercially available from Life Technologies, Incorporated.

The resulting RT products were then amplified via polymerase chain reaction ("PCR") using about a 0.5 unit (per 100  $\mu$ l reaction) of a thermostable DNA polymerase which is commercially available from Perkin-Elmer-Cetus Corporation under the tradename "Taq polymerase" and about 0.1  $\mu$ mol/reaction of gene-specific primers available from Clontech Laboratories, Inc. ("Clontech") or designed as set forth in Table 2 in accordance with the procedures set forth in the protocol accompanying the primers from Clontech for mouse glyceraldehyde-3-phosphate-dehydrogenase (G3PDH), transcription factors and cytokines, or as described in Table 2.

Table 2 illustrates some of the DNA primers used, the amount of  $MgCl_2$  required for the PCR reaction, and the length of the PCR cycle.

**Table 2: DNA Primers Utilized in RT-PCR Assay\*\*\***

Primer (See attached Sequence Listing)	Amt. of $MgCl_2$ (mM)	Cycle (min) @ °C	No. of cycles	DNA Seq. ID No.
--	--------------------------------	------------------------	------------------	--------------------------

Transglutaminase sense AACCCCAAGT TCCTGAAG	2.5	1 @ 94; 2 @ 55; 3 @ 72	35	1
Transglutaminase antisense TTTGTGCTGG GCCACTTC	2.5	1 @ 94; 2 @ 45; 3 @ 72	35	2
Tyrosinase sense TCAGCCCAGC ATCCTTCTTC	5	1 @ 94; 2 @ 45; 3 @ 72	35	3
Tyrosinase antisense CAGCCATTGT TCAAAAATAC TGTCC	5	1 @ 94; 2 @ 55; 3 @ 72	35	4
Proopiomelanocortin ("POMC") sense AAAAGAAGAG AGAAGAGCGA C	2.5	1 @ 94; 2 @ 55; 3 @ 72	35	5
POMC antisense AGAGCTGAGA CACCTTACC	2.5	1 @ 94; 2 @ 55; 3 @ 72	35	6
Collagenase sense AAGACCCCAA CCCTAAGCAC	2.5	1 @ 94; 2 @ 53; 3 @ 72	35	7
Collagenase antisense CAGCACTGAC GGTTTTACAC	2.5	1 @ 94; 2 @ 53; 3 @ 72	35	8

5

The PCR products were then analyzed by 2% agarose/ethidium bromide gels and analyzed according to methods well-known in the art in order to compare the level of expression of certain genes during the hair

5 cycle of both trypsin-treated and untreated mice. When necessary for better visualization, the resulting PCR products were precipitated with ethanol according to well-known procedures. When primers for G3PDH were used, only 10% of the PCR reaction products were used. An RNA sample from the skin of a C57BL/6 female, 7 to 8 week old mouse that was not reverse-  
10 transcribed was used as a negative control for each PCR amplification. An RNA sample from the skin of a six month old C57BL/6 female mouse having a non-synchronized hair cycle was used as a positive control when positive controls were not commercially available. The migration of the RT-PCR products on the gels was always identical to that of the positive controls,  
15 and to that of the reported amplicon sizes.

The relative quality of each respective RT-PCR reaction product was then compared by analyzing the mRNA level of G3PDH, a "housekeeping" gene, in each respective product. As illustrated in FIG. 5, G3PDH gene expression was found to be similar at all the time points  
20 examined, which thereby enabled the comparison of the relative levels of gene expression for the desired genes.

To verify that the delay in follicular development was not the result of a non-specific irritation or inflammatory response, we analyzed the mRNA levels of genes that are typically upregulated during such situations. After  
25 examination of the mRNA levels of genes from the RT-PCR products, we found that there was no change in the mRNA levels of IL-6, IL-10 and GM-CSF, a slight upregulation of  $TNF\alpha$ , a slight down regulation of  $TNF\beta$  and  $TNF-R1$ , and a moderate down regulation in Macrophage Inducible Protein (MIP) as illustrated in Table 3.

5

**Table 3: Patterns of Gene Expression**

<b>Gene</b>	<b>Untreated</b>	<b>Trypsin-Treated</b>
IL-6	-	-
IL-10	+/-	+/-
GM-CSF	-	-
TNF $\alpha$	+/-	+
TNF $\beta$	+	+/-
TNF-RI	+/-	+/- -
TNF-RII	-	-
MIP	+	-
IL-1 $\alpha$	+	++
IL-1 $\beta$	+/-	+++
IL-1R	-	-
IFN $\gamma$	-	+++
c-myc	+	-
c-myb	++	+
c-fos	+	+/-
c-jun	+/-	+
Collagenase	+	+/-
Tyrosinase	+++	+/-
POMC	+	+/-
Transglutaminase	+	+

\* Note that RT-PCR is only semi-quantitative. Comparisons are valid only for each amplified sequence within the different hair cycles, and not among the different genes.

-	no detectable expression	+	a strong band
+/- -	a very weak band	++	a stronger band
+/-	a weak band	+++	a very strong band

10

5 The profile of gene expression in Table 3 suggests against an inflammatory reaction or a response to dermal irritation.

A moderate upregulation was observed in IL-1 $\alpha$  mRNA level (FIG. 5b & Table 3), a gene associated with the inhibition of hair growth in culture. Since the 1L-1 $\alpha$  induction could have also resulted from the loss of the epidermal barrier function, we analyzed the barrier integrity by measuring transepidermal water loss (TEWL). TEWL was measured with an "Evaporimeter EPI" evaporimeter available from Servomed AB by first normalizing the evaporimeter with the ambient humidity and then placing the probe on the dorsal skin of the test subject. The results showed only a moderate increase in TEWL which did not correlate with trypsin concentration as shown in Table 4.

**Table 4: TEWL in Trypsin Treated Skins**

<u>Treatment</u>	<u>Sample Size</u>	<u>TEWL</u>
Untreated	6	27.01 $\pm$ 3.8
0.01% Trypsin	4	36.89 $\pm$ 4.6
0.1% Trypsin	4	43.2 $\pm$ 5.2
0.5% Trypsin	4	34.53 $\pm$ 4.9
1% Trypsin	4	37.5 $\pm$ 3.9
1% Trypsin - inactive (mild)	3	33.99 $\pm$ 6.2

20 Therefore, it is likely that any upregulation of IL-1 $\alpha$  in our RT-PCR product as illustrated in Table 3 and FIG 5 is associated with the inhibition of hair growth.

As illustrated in FIGS. 5a-b & Table 3, a stronger increase in mRNA levels was demonstrated for 1L-1 $\beta$  and IFN $\gamma$ , which Are both known to be upregulated in Alopecia Areata and to be associated with the inhibition of hair growth. This further suggested that the only inflammatory-associated

5 genes upregulated by trypsin were those associated with inhibition of hair growth.

As illustrated in Table 3, a slight reduction was detected in the mRNA levels of *c-myb*, *c-myc* and *c-fos*, while *c-jun* level was slightly increased throughout the delay period. Collagenase, a gene regulated via an AP-1  
10 response element, was slightly reduced as well. This observation of a general slight reduction in mRNA levels of several genes was further reflective of the overall slow down in hair growth of trypsin-treated skin.

As illustrated in FIG. 5A and Table 3, tyrosinase, a key enzyme for hair pigmentation, was downregulated during the delay in hair growth  
15 period, while its mRNA level increased as the follicles started to overcome the delay. POMC, the precursor of the melanogenic peptide melanocyte stimulating hormone (MSH), was moderately downregulated throughout the delay period as shown in FIG. 5A and Table 3. The downregulation of these two genes indicated that trypsin also affected the regulation of hair  
20 pigmentation.

This Example showed that the trypsin perturbation of the hair cycle may be understood by examination of the expression pattern of a series of genes throughout the delay period. Trypsin-induced changes in mRNA levels along the hair cycle were clearly evidenced, indicating a regulatory  
25 role for trypsin in hair growth and hair pigmentation. Moreover, this Example further reflected the specificity of the topical application of trypsin on hair growth and hair pigmentation genes.

#### **EXAMPLE 8: Other Serine Proteases Which Affect Hair Growth**

30 A single topically active composition as prepared in Example 2 was applied to the skin surface of C57BL/6 female, 7 to 8 week old mice after hair induction in accordance with the method set forth in Example 3.



5 The procedure of Example 3 was repeated, but with the substitution of carboxypeptidase-Y, protease IV, and subtilysin, respectively, for the trypsin.

After 8 days post-treatment, the skin of each respective mouse was examined using a Minolta Chromameter Model CR300 chromameter in order to compare their respective skin colors.

10 We observed that trypsin, a member of the endopeptidase family which cuts a protein at the C-side of Arginine and Lysine, induced the longest delay in hair growth. By contrast, the carboxypeptidase-Y, which hydrolyzes L-amino acids at the C-termini of proteins, and protease IV, a member of the nonspecific endopeptidase family which cuts 56% of peptide bonds at neutral pH, had only minimal delaying effect, while subtilysin, a member of the nonspecific peptidase family which cuts peptide bonds at alkaline pH, only slightly increased the rate of hair growth. The results of this Example are illustrated in Table 5.

20 **Table 5: Color Measurements of Serine Protease Treated Skins**

<u>Treatment</u>	<u>Sample Size</u>	<u>L*</u>
Untreated	6	49 ± 0.30
1% Trypsin	6	56.1 ± 0.81
1% Subtilysin	4	42.9 ± 2.87
1% Endopeptidase	4	51.8 ± 0.11
1% Carboxypeptidase-Y	4	51.9 ± 0.47

(The L\* scale: 0 = black and 100 = white, is a measure of hair pigmentation as viewed from the skin surface.)

As illustrated in Table 5, the trypsin-treated hair follicles were the least developed and thus appeared to impart the lightest color to the skin. This Example suggests that the appearance of skin color is related to hair development in the hair follicle.

5           Since the results obtained from the trypsin-treated skins showed  
trypsin to be relatively superior at delaying hair growth and hair  
pigmentation, this Example supports the belief that the effect of trypsin on  
hair growth and hair pigmentation may not be exclusively the result of a  
non-specific proteolytic digestion, but rather it may also be the result of a  
10       specific activity induced by trypsin that causes the activation of the  
programmed cell death pathway.

**EXAMPLE 9: Trypsin Affects an Early Step in Hair Growth Induction**

15           A single topically active composition as prepared in Example 2 was  
applied to the skin surface of 3 C57BL/6 female, 7 to 8 week old mice after  
hair induction in accordance with the method set forth in Example 3. This  
procedure was repeated on nine additional mice, but the treatment was  
applied either two, three or seven times per week, respectively.

20           After observing the skin color of the mice for 8 days, the skin of the  
treated mice was lighter than that for the untreated controls. This further  
suggested that the trypsin-treated hair follicles were the least developed  
and thus appeared to impart the lightest color to the skin.

25           1 cm x 2 cm samples of the skins of the respectively-treated mice  
were obtained, H&E stained, then examined histologically via the procedure  
set forth in Example 1. Histological analysis of the stained samples  
supports the fact that the additional daily treatments of the animals with  
trypsin did not prolong the delay in hair growth. This indicated that the  
induction of cell death by a serine protease resulted from a specific and  
timely event.

30           In order to determine the timing of this specific event, a single  
topically active composition as described in Example 2 was applied to the  
skin surface of C57BL/6 female, 7 to 8 week old mice after hair induction in

accordance with the method set forth in Example 3, except that the treatment was applied at various time intervals as indicated in Table 6.

As illustrated in Table 6, the skin color chromametric measurements obtained in accordance with the method set forth in Example 8 demonstrated an increase in darkness (more pigment, less of the delay) that correlated with the increased time between hair growth induction and trypsin application. Mice treated immediately after hair growth induction showed the longest delay in hair growth and hair pigmentation. Mice treated 2 hours and 4 hours after hair growth induction also exhibited a delayed, but progressively shorter hair cycle. Mice treated 6 or more hours after depilation were not delayed for hair growth and were indistinguishable from the untreated control.

Table 6: Color Measurements of Trypsin Treated Skin\*\*

<u>Treatment</u>	<u>Sample Size</u>	<u>L*</u>
Untreated	6	49 ± 0.32
1% Trypsin, immediately	6	56.1 ± 1.54
1% Trypsin, after 2 hours	4	54.4 ± 1.30
1% Trypsin, after 4 hours	4	53.3 ± 1.23
1% Trypsin, after 6 hours	4	48.0 ± 2.01
1% Trypsin, after 18 hours	4	49.2 ± 1.07
1% Trypsin, after 48 hours	4	50.4 ± 1.01

\*\* Skin samples were tested 8 days post-depilation.

This Example shows that the application of trypsin to the skin is effective for delaying hair growth and hair pigmentation when applied within about 4 hours, and preferably immediately after hair growth induction.

#### EXAMPLE 10: *The Trypsin Effect May*

### Involve a Receptor-Mediated Pathway

A single topically active composition as prepared in Example 2 was applied to the skin surface of C57BL/6 female, 7 to 8 week old mice after hair induction in accordance with the method set forth in Example 3, with the exception that the concentration of the trypsin was varied between 1% (w/v) to 0.01% (w/v) ( $4 \times 10^{-4}$  M -  $4 \times 10^{-6}$  M). At 9 days post-depilation, the mice were analyzed colorimetrically via the procedure set forth in Example 9.

As illustrated in Table 7, the results of the colorimetric analysis showed an increase in brightness ( $L^*$ , more white, less pigment) which correlated with a decrease in trypsin concentration and with an increase in the delay of the hair cycle.

**Table 7: Skin Brightness of Trypsin Treated Skins\*\***

<u>Treatment</u>	<u>Sample Size</u>	<u><math>L^*</math></u>
Untreated	6	$45 \pm 0.97$
0.01% Trypsin	4	$48.6 \pm 1.01$
0.1% Trypsin	4	$49.5 \pm 1.07$
0.5% Trypsin	4	$47.7 \pm 0.3$
1% Trypsin	4	$47.9 \pm 0.08$
1% Trypsin - inactive (mild)	3	$52.2 \pm 0.17$

$L^*$  scale: 0 = black, 100 = white

\*\* Samples were analyzed 9 days post-depilation.

This Example suggests the presence of a receptor-mediated mechanism including desensitization with higher doses.

### **EXAMPLE 11: The Proteolytic Activity of Trypsin is Unnecessary for Delaying Hair Growth**

5           After the composition of Example 2 was incubated at room temperature for 48 hours, its proteolytic activity was analyzed via a serine protease activity detection kit available from PanVera, Corporation, which showed that the preparation had no detectable proteolytic activity. The inactive composition was applied to the skin surface of 3 C57BL/6 female, 7  
10 to 8 week old mice after hair induction in accordance with the method set forth in Example 10. At 9 days post-depilation, the mice were analyzed colorimetrically via the procedure set forth in Example 8. As illustrated in Table 7, it was evident that the lack of proteolytic activity of trypsin did not reduce the activity of this preparation in delaying hair growth.

15           This Example was repeated, except that the incubated trypsin was substituted with trypsin boiled for 10 minutes at temperature of 100 °C. The results showed that the use of boiled trypsin, which was proteolytically inactive as well, had no delaying effect on hair growth. The act of boiling, which denatured the three-dimensional structure of trypsin, suggested that  
20 the three-dimensional structure of trypsin, and not just its proteolytic activity, contributed to the delaying effect on the hair cycle. Regardless of whether the trypsin blocked a survival signal or activated a death receptor to induce this process, this Example suggested that the configuration of the trypsin molecule might also have been responsible for the induction of programmed  
25 cell death and apoptosis in the follicular papillae.

#### **EXAMPLE 12: Use of Trypsin-Containing Composition**

          Glycerol dilaurate/cholesterol/polyoxyethylene-10-stearyl ether liposomes are prepared in accordance with the procedures set forth in  
30 Niemiec, wherein the constituent compounds of the liposomes are in a ratio of about 58:15:27, respectively.

          After cooling the resulting liposomes to a temperature of about 40 °C, the liposomes are added with normal mixing to an amount of a thickening

5 agent composed of polyacrylamide (and) C13-C14 isoparaffin (and) laureth-  
7, available from Seppic, Inc. under the trade name "SEPIGEL 305," such  
that the amount of the thickening agent in the final composition is about 1-  
3% (w/v).

10 A sufficient amount of trypsin is then added thereto to produce a 1%  
trypsin (w/v) composition. This composition is appropriate for immediate  
topical application.

The resulting product possesses characteristics and properties of an  
effective shaving gel.

5           We claim:

1.       A method for affecting changes in mammalian hair growth and hair pigmentation comprising:

          topically applying to skin of a mammal an effective amount of a topically active composition comprising a protease within a time sufficient  
10       relative to telogen hair growth induction.

2.       The method of claim 1 wherein the protease is a serine protease.

3.       The method of claim 2 wherein the protease is selected from trypsin, carboxypeptidase-Y, protease IV, subtilysin or mixtures thereof.

4.       The method of claim 3 wherein the protease is trypsin.

15       5.       The method of claim 4 wherein the protease is present in an amount, based upon the total volume of the topically active composition, of from about 0% (w/v) to 5% (w/v).

6.       The method of claim 5 wherein the protease is present in an amount, based upon the total volume of the topically active composition, of from  
20       about 0.01% (w/v) to about 1% (w/v).

7.       The method of claim 1 wherein one of said changes is a delay in hair growth and hair pigmentation.

8.       The method of claim 1 wherein said topically active composition further comprises a pharmaceutically or cosmetically acceptable vehicle.

25       9.       The method of claim 8 wherein said pharmaceutically or cosmetically acceptable vehicle is a liposome or mixture thereof.

10.       The method of claim 9 wherein said liposome is non-ionic.

11.       The method of claim 10 wherein said liposome is comprised of:

          a) glycerol dilaurate, glycerol distearate, or a mixture thereof;

30       b) a compound having a steroid backbone as found in cholesterol or a mixture thereof; and

          c) a fatty acid ether having from about 12 to about 18 carbon atoms or a mixture thereof.

- 5           12.    The method of claim 10 wherein said liposome is comprised of:  
            a) glycerol dilaurate;  
            b) cholesterol; and  
            c) polyoxyethylene-10-stearyl ether.
- 10           13.    The method of claim 11 wherein the components of said liposome are  
            present in a ratio of about 53:10:22 to about 63:20:32, respectively.
14.    The method of claim 8 wherein said pharmaceutically or cosmetically  
            acceptable vehicle is present in an amount, based upon the total volume of  
            said topically active composition, of from about 0 mg/mL to about 100  
            mg/mL.
- 15           15.    The method of claim 1 wherein the hair growth induction of the  
            telogen hair is performed by a method comprising shaving, electrolysis, wax  
            depilation, chemical depilation or combinations thereof.
16.    The method of claim 1 wherein said time is immediately before,  
            immediately after or simultaneously with said hair growth induction of  
20           telogen hair follicles.
17.    The method of claim 16 wherein said time is either simultaneously  
            with or immediately following hair growth induction.
18.    The method of claim 1 wherein the composition further comprises  
            surfactants, moisturizers, humectants, conditioners, fragrances, colorants,  
25           or mixtures thereof.
19.    The method of claim 18 wherein said composition is in the form of  
            shaving creams, shaving gels, shaving powders, chemical depilatory  
            creams, other products whose purpose is to either facilitate the removal of  
            hair or actually remove hair or combinations thereof.
- 30           20.    The method of claim 1 further comprising leaving said composition on  
            said skin for a period sufficient to affect said changes.
21.    The method of claim 20 wherein said period is at least about fifteen  
            minutes.



5 22. The method of claim 1 further comprising mixing said protease with a buffer comprising N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid, (hydroxymethyl) aminomethane or mixtures thereof.

23 A method for inducing programmed cell death and apoptosis within a follicular papilla of a mammal comprising:

10 topically applying to skin of the mammal a topically active agent having a first portion which is similar in shape to a second portion of a three-dimensional structure of trypsin, whereby both said first portion and said second portion are capable of receptor-mediated signal transduction.

24. The method of claim 23 wherein said agent is trypsin, proteolytically inactive trypsin or combinations thereof.

15 25. A composition comprising:

a) a topically active agent having a first portion which is similar in shape to a second portion of a three-dimensional structure of trypsin, whereby both said first portion and said second portion are capable of receptor-mediated signal transduction; and

20 b) a pharmaceutically or cosmetically acceptable vehicle.

26. The composition of claim 25 wherein the topically active agent is trypsin, proteolytically inactive trypsin or combinations thereof.

27. The composition of claim 25 wherein the topically active agent is present in an amount, based upon the total volume of the composition, of from about 0% (w/v) to about 5% (w/v).

28. The composition of claim 25 wherein the topically active agent is present in an amount, based upon the total volume of the composition, of from about 0.01% (w/v) to about 1% (w/v).

29. The composition of claim 25 wherein the pharmaceutically or cosmetically acceptable vehicle is a liposome.

30. The composition of claim 29 wherein said liposome is non-ionic.

31. The composition of claim 30 wherein said liposome is comprised of:

- 5           a) glycerol dilaurate, glycerol distearate, or a mixture thereof;  
          b) a compound having a steroid backbone as found in cholesterol or  
a mixture thereof; and  
          c) a fatty acid ether having from about 12 to about 18 carbon atoms  
or a mixture thereof.
- 10       32.    The composition of claim 30 wherein said liposome is comprised of:  
          a) glycerol dilaurate;  
          b) cholesterol; and  
          c) polyoxyethylene-10-stearyl ether.
- 15       33.    The composition of claim 31 wherein the components of said  
liposome are present in a ratio of about 53:10:22 to about 63:20:32,  
respectively.
- 20       34.    The composition of claim 25 wherein the pharmaceutically or  
cosmetically acceptable vehicle is present in an amount, based upon the  
total volume of composition, of from about 0 mg/mL to about 100 mg/mL.
- 25       35.    The composition of claim 25 wherein the composition further  
comprises surfactants, moisturizers, humectants, foaming agents, cosmetic  
adjuvants, buffering agents, preservatives, anti-oxidants, conditioners,  
fragrances, colorants, or mixtures thereof.
- 30       36.    The composition of claim 35 wherein said composition is in the form  
of shaving creams, shaving gels, shaving powders, chemical depilatory  
creams, other products whose purpose is to either facilitate the removal of  
hair or actually remove hair or combinations thereof.
- 35       37.    A composition comprising:  
          a) a topically active agent comprising a protease capable of inducing  
apoptosis in a follicular papilla; and  
          b) a pharmaceutically or cosmetically acceptable vehicle.
- 40       38.    The composition of claim 37 further comprising a component selected  
from surfactants, moisturizers, humectants, foaming agents, cosmetic

5       adjuvants, buffering agents, preservatives, anti-oxidants, conditioners,  
          fragrances, colorants, or mixtures thereof .

39.    The composition of claim 37 wherein said protease is a serine  
          protease.

40.    The composition of claim 39 wherein said protease is selected from  
10       trypsin, carboxypeptidase-Y, protease IV, subtilysin or mixtures thereof.

41.    The composition of claim 37 wherein the topically active agent is  
          present in an amount, based upon the total volume of the composition, of  
          from about 0% (w/v) to about 5% (w/v).

42.    The composition of claim 37 wherein the topically active agent is  
15       present in an amount, based upon the total weight of the composition, of  
          from about 0.01% (w/v) to about 1% (w/v).

43.    The composition in accordance with claim 37 wherein the  
          pharmaceutically or cosmetically acceptable vehicle is a liposome.

44.    The composition in accordance with claim 43 wherein said liposome  
20       is non-ionic.

45.    The composition of claim 43 wherein said liposome is comprised of:

          a) glycerol dilaurate, glycerol distearate, or a mixture thereof;

          b) a compound having a steroid backbone as found in cholesterol or  
          a mixture thereof; and

25       c) a fatty acid ether having from about 12 to about 18 carbon atoms  
          or a mixture thereof.

46.    The composition of claim 43 wherein said liposome is comprised of:

          a) glycerol dilaurate;

          b) cholesterol; and

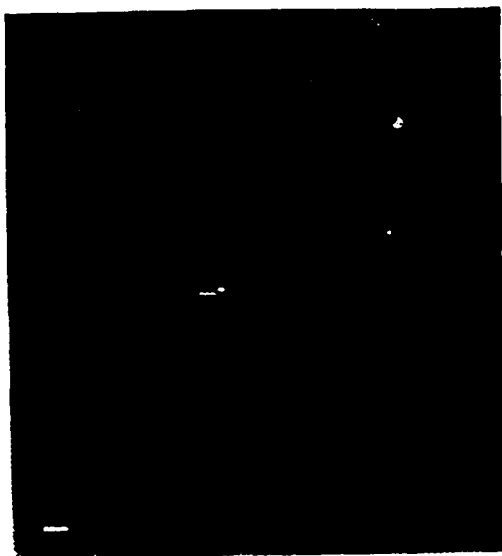
30       c) polyoxyethylene-10-stearyl ether.

47.    The composition of claim 45 wherein the components of said  
          liposome are in a ratio of about 53:10:22 to about 63:20:32, respectively.

5        48.    The composition of claim 37 wherein the pharmaceutically or  
cosmetically acceptable vehicle is present in an amount, based upon the  
total volume of composition, of from about 0 mg/mL to about 100 mg/mL.

49.    The composition of claim 37 wherein said composition is in the form  
of shaving creams, shaving gels, shaving powders, chemical depilatory  
10    creams, other products whose purpose is to either facilitate the removal of  
hair or actually remove hair or combinations thereof.

**FIG. 1A**



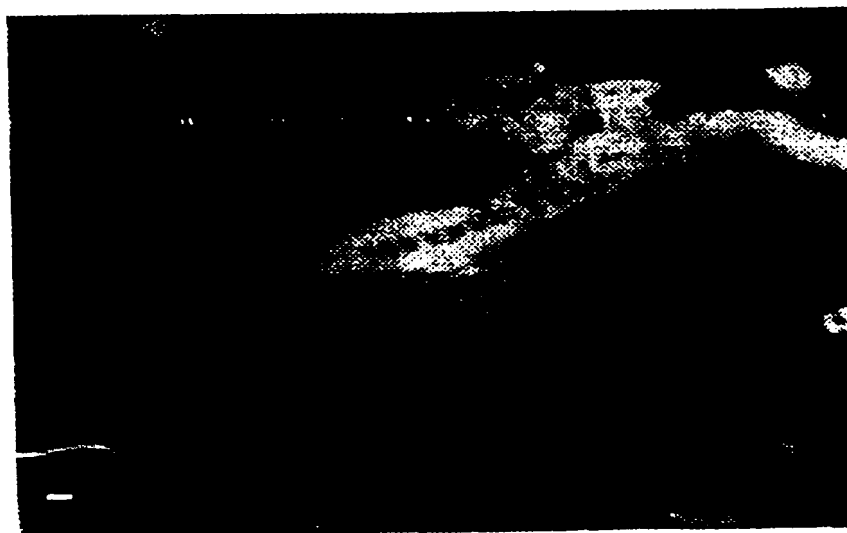
Bar=10 $\mu$ m

**FIG. 1B**



Bar=10 $\mu$ m

**FIG. 1C**



Bar=10 $\mu$ m

**FIG. 2A**



**FIG. 2B**



**FIG. 2C**



**FIG. 3A(a)**Bar=10 $\mu$ m**FIG. 3A(b)**Bar=10 $\mu$ m**FIG. 3A(c)**Bar=10 $\mu$ m**FIG. 3A(d)**Bar=5 $\mu$ m

**FIG. 3B(a)**



Bar=10μm

**FIG. 3B(b)**



Bar=10μm

**FIG. 3B(c)**



Bar=10μm

**FIG. 3B(d)**



Bar=10μm

**FIG. 3B(e)**



Bar=10μm

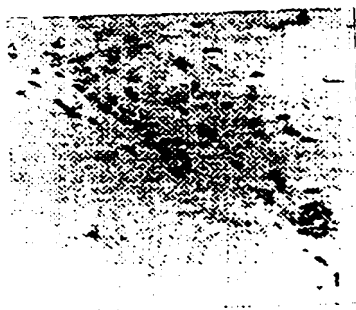
**FIG. 3B(f)**



Bar=5μm

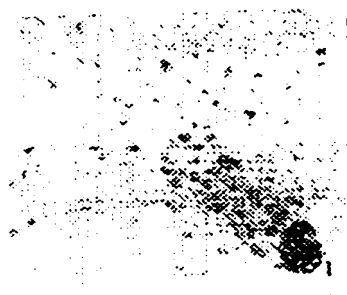


**FIG. 4A(c)**



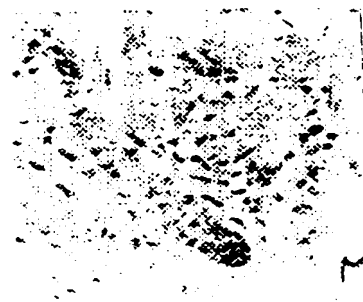
Bar=10µm

**FIG. 4A(b)**



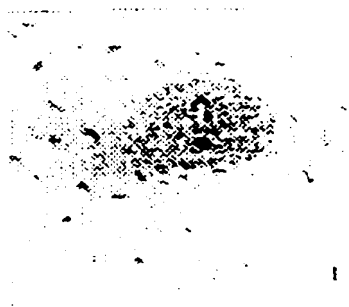
Bar=10µm

**FIG. 4A(a)**



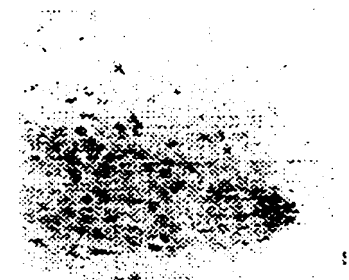
Bar=10µm

**FIG. 4B(a)**



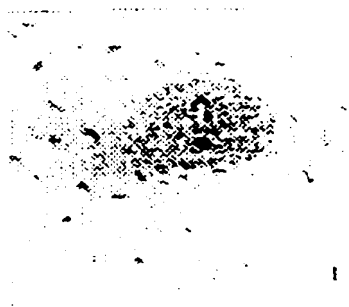
Bar=10μm

**FIG. 4B(b)**



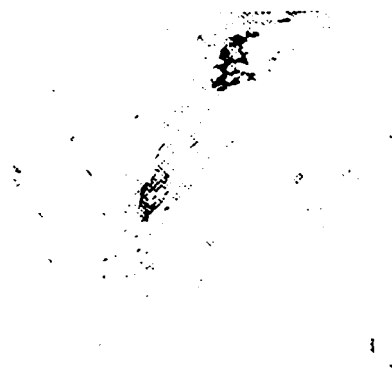
Bar=5μm

**FIG. 4B(c)**



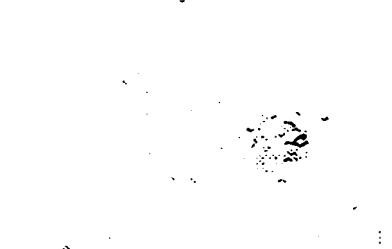
Bar=5μm

**FIG. 4B(f)**



Bar=5μm

**FIG. 4B(e)**

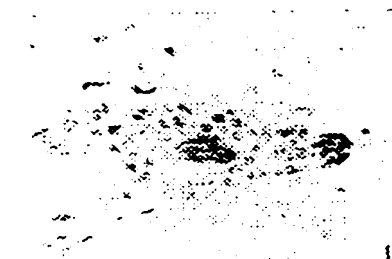


Bar=10μm

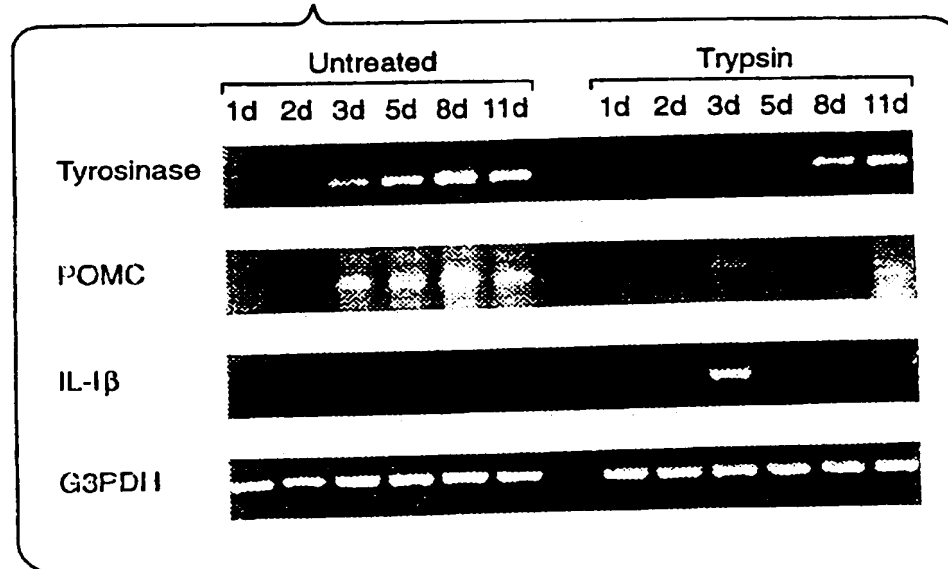
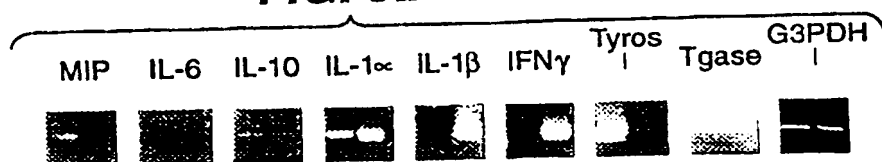
**FIG. 4B(d)**



**FIG. 4B(d)**



Bar=10μm

**FIG. 5A****FIG. 5B**

# INTERNATIONAL SEARCH REPORT

International Application No.

PCT/US 97/11033

**A. CLASSIFICATION OF SUBJECT MATTER**  
IPC 6 A61K7/06

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P, X	WO 96 25946 A (MASSACHUSETTS INST TECHNOLOGY ; HORVITZ H ROBERT (US); YUAN JUNYING) 29 August 1996 see page 23, line 29 - line 37 see page 44, paragraph 1 ---	1
X	WO 93 25694 A (MASSACHUSETTS INST TECHNOLOGY) 23 December 1993 see page 14, line 23 - line 32 see page 17, line 26 - line 28 see page 18, line 20 - line 31 ---	1
A	EP 0 079 074 A (AYUKAWA TAIZO ; KAKUDAI SHOSAN KK (JP)) 18 May 1983 see the whole document ---	1
-/--		

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

**Special categories of cited documents:**

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claims or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

- "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- "X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- "Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- "&" document member of the same patent family

Date of the actual completion of the international search

14 November 1997

Date of making of the international search report

03/12/1997

Name and mailing address of the ISA

European Patent Office, P. B. 5818 Patentlaan 2  
NL - 2280 HV Rijswijk  
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl  
Fax: (+31-70) 340-3016

Authorized officer

Couckuyt, P

# INTERNATIONAL SEARCH REPORT

International Application No.

PCT/US 97/11033

## C. (Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication where appropriate, of the relevant passages	Relevant to claim No.
A	CHEMICAL ABSTRACTS, vol. 120, no. 7, 14 February 1994 Columbus, Ohio, US; abstract no. 75653, XP002046977 & JP 05 276 939 A (NOEVIR KK) 26 October 1993 see abstract -----	1
P, A	DATABASE MEDLINE US NATIONAL LIBRARY OF MEDICINE (NLM), BETHESDA, MD, US SEIBERG M ET AL: "Trypsin-induced follicular papilla apoptosis results in delayed hair growth and pigmentation." XP002046978 & DEVELOPMENTAL DYNAMICS, vol. 208, no. 4, - April 1997 pages 553-64, see abstract -----	1

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 97/11033

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9625946 A	29-08-96	AU 5133696 A	11-09-96
WO 9325694 A	23-12-93	EP 0672151 A	20-09-95
		JP 8500482 T	23-01-96
		WO 9325685 A	23-12-93
EP 0079074 A	18-05-83	JP 58079913 A	13-05-83
		CA 1187416 A	21-05-85
		US 4508707 A	02-04-85